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Ultrastructural and ontogenetic aspects of the "inner anther tapetum" in *Rhinanthus* (Scrophulariaceae)

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**Key words:** Rhinanthus, Scrophulariaceae. - Anther tapetum, differentiation, polyploidization, endomitosis.

Abstract

It has been stated formerly that polyploidization in the anther tapetum of Rhinanthus occurs by two distinct processes: initially a normal mitosis may occur, leading to binucleate cells, then endomitoses (exact sense) follow. Additionally we now present evidence for the occurrence of arrested mitoses during the development of the inner tapetum based on light microscopical observations and an ultrastructural study of dumb-bell shaped nuclei (products of anaphase arrest). The present results warrant the conclusion that mitotic blocks are regular events of the early premeiotic period, but are restricted to the inner tapetum. They lead to polyenergid cells and also polyploid nuclei already at a time when the anther wall and the outer tapetum is just in the process of differentiation by periclinal divisions. The concept of normal mitosis/endomitosis for the tapetum development in Rhinanthus holds for the meiotic period, however. As a working hypothesis, a causal explanation of the differential development of inner and outer tapetum is presented, and the systematic relevance of tapetum cytology in Pediculariaceae is discussed.

### Introduction

The present investigation concerns some ontogenetic and ultrastructural aspects of the anther tapetum in Rhinanthus, and continues recent studies about the cytology of the anther tapetum in the Pedicularieae (TSCHERMAK-WOESS 1967, GREILHUBER 1974). These studies especially had in view the distinction and different occurrence of endomitosis (exact sense, see GEITLER 1953, TSCHERMAK-WOESS 1971, NAGL 1973) and formation of restitution nuclei.

Endomitosis resp. formation of restitution nuclei must be considered to be two qualitatively different modes producing polyploid nuclei and therefore are important for the typological classification of the anther tapetum in general (CARNIEL 1952, 1963, TSCHERMAK-WOESS 1967, GREILHUBER 1974). Both processes do occur in certain Pedicularieae, but in a distinct pattern specific for each genus (GREILHUBER 1974). In this regard the development of the inner tapetum reveals remarkable features. While the outer tapetum regularly remains uninucleate diploid until meiosis has started, the inner tapetum becomes polyploid and partially bi- or multinucleate already early in the premeiotic period in most genera (NIKITICHEVA 1968, GREILHUBER 1974). This leads to a remarkable dimorphism of the tapetum.

In the glandular tapetum of Rhinanthus evidence for endomitotic polyploidization of nuclei has been given by (TSCHERMAK-WOESS 1967). The tapetum consists of uni- and binucleate cells, the latter arising by normal acytokinetic mitoses during the early meiotic period. Then endomitoses follow and bring about endopolyploid uni- and binucleate cells. With a view to the peculiar behaviour of the inner tapetum in most of the related genera we were

wondering whether premeiotic polyploidization of the inner tapetum may be absent in Rhinanthus or not. In this paper we present observations concerning this question together with some considerations about the causes of the developmental differences between the inner and the outer tapetum.

### Material and methods

Rhinanthus alectorolophus (Scop.) Poll. was collected at Lunz (Lower Austria). Young buds were fixed in acetic-ethanol or in phosphate-buffered glutaraldehyde (4%, pH 7,2). For cytological examination in the light microscope the carmine squash technique was employed and both fixatives gave suitable results (after fixation in glutaraldehyde the nuclear contour appears more clearly pronounced than after AE-fixation and the protoplasts remain unaltered during squashing). For EM-observation glutaraldehyde-fixed anthers of light-microscopically examined buds were post-fixed in  $\text{OsO}_4$ , embedded (after SPURR 1969) and thin sectioned. Micrographs were taken with a ZEISS EM 9A. For light microscopical observations also semi-thin sections were stained with toluidin blue.

### Results and discussion

#### Origin of sporogenous cells, wall layers, and tapetum.

Our observations about sporangium development in Rhinanthus completely are in accordance with the findings of NIKITICHEVA (1968) in the related genera Bartsia, Lathraea, and Melampy-

rum, and to our earlier results in Bellardia, Euphrasia, Odontites, Pedicularis, and Tozzia (GREILHUBER 1974, and unpublished results). Thus, the origin of the archesporium and the development of the anther wall are seemingly identical within the tribus Pedicularieae. The primary archesporium is a crooked monolayered plate of subepidermal cells, which divides into secondary archesporium (= primary sporogenous cells), which gives rise to the pollen mother cells, and primary parietal layer. The latter divides into two secondary parietal layers, the inner one already representing the outer tapetum; the outer one again divides into endothecium and middle layer. Wall formation therefore is according to the dicotyledonous type (see DAVIS 1966). The inner tapetum directly derives from meristematic connective cells which lie adjacent to the inner side of the crooked primary archesporium. Clearly, in this case inner and outer tapetum are of different origin and must be regarded to be inhomologous entities. This dual origin of the tapetum is long known (see SCHNARF 1929, pp. 1, 39) but nearly always neglected in studies about tapetum development, and much vagueness exists about the exact origin of the inner tapetum. Recently PERIASAMY and SWAMY (1966) have pinpointed these problems. According to their opinion the dual origin of the tapetum ("normal type") is masked by equal function and development during sporogenesis. Only in certain cases the inherent difference becomes visible by a promoted development of the inner tapetum (larger cells, larger or more nuclei, more layers), which is then called "inner tapetum" properly.

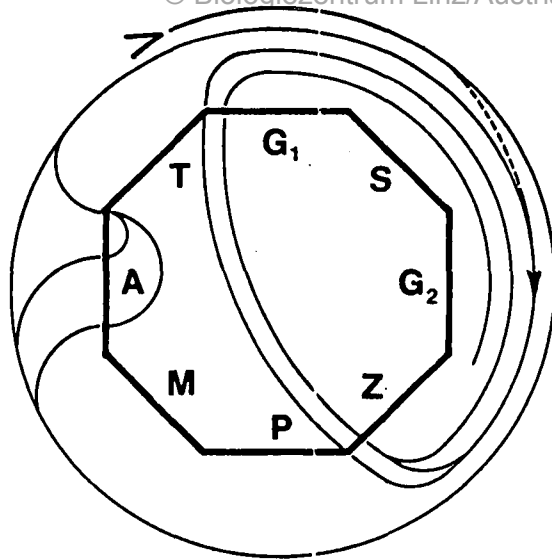
#### Cytology of the tapetum.

In general, from a certain stage of tapetum development on cytokineses do not occur any more. However, duplication of chromosomes continues, giving rise to polyploid cells. Dependent on whether karyokinesis is completed or not, or

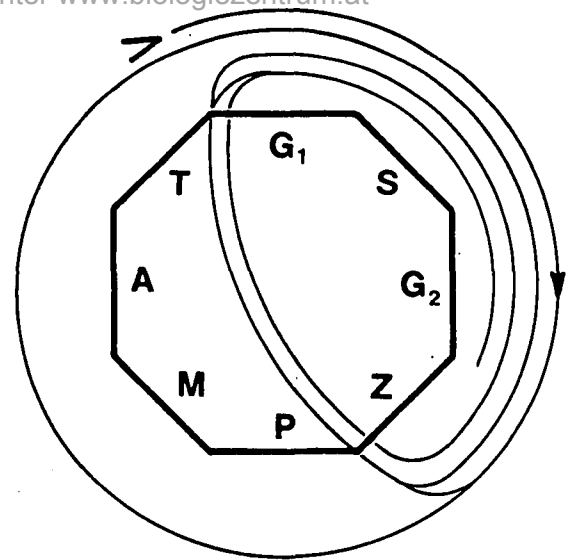
initiated at all, uni- or multinucleate cells arise. In this regard two different modes of polyploidization must be distinguished. The first consists in formation of restitution nuclei, i.e. mitosis starts but is disturbed and arrested in any stage between an early stage of prophaseic spiralization and telophase, and interphase nuclei are reconstituted. Further, neighbouring anaphase plates or spindles may fuse to bring about polyploid nuclei. This is the most common origin of polyploid nuclei in the anther tapetum of the angiosperms. The second mode is endomitosis of the angiospermous type (exact sense), i.e. an exactly regulated and well defined "short cut" of the nuclear cycle without any prophaseic spiralization of the chromosomes. The only visible structural change during endomitosis of angiosperms is a dispersion of the chromocenters into chromomere like granules when the nuclei pass from one level of ploidy to the other. This endomitotic structural change is called "endomitotic dispersion stage" ("endomitotisches Zerstäubungsstadium") and is equivalent to the "mitotic dispersion stage" which is the first visible structural change when nuclei leave interphase and enter mitosis (HEITZ 1929).

As a rule, the mode of polyploidization is species specific or even genus specific, i.e., in a given species the tapetum either enlarges by formation of restitution nuclei or by endomitoses (CARNIEL 1952, 1963, TURALA 1963, TSCHERMAK-WOESS 1967). However, the present together with recent results in Pedicularieae (GREILHUBER 1974) show, that the features can be more complicated than believed earlier.

Our karyological results in Rhinanthus are schematically presented in Fig. 1. Evidently some remarkable differences exist between the development of the inner and the outer tapetum, which have their origin at the time of anther wall differentiation and generation of the outer tapetum. Of course, sporogenous cells are still mitotically active



**Inner tapetum**



**Outer tapetum**

**Fig. 1.** Schematic representation of the possible nuclear cycle passages in the anther tapetum of Rhinanthus after the last cytokinesis (with reference to the schema of NAGL 1973).  $G_1$  = presynthetic gap, S = synthetic period,  $G_2$  = postsynthetic gap, Z = dispersion stage, P = prophase (spiralization stage), M = metaphase, A = anaphase, T = telophase; open pin = start of acytokinetic cycles, black pin = start of the meiotic period, dotted line = rare and accidentally occurring nuclear pathway.

("premeiotic period" after CARNIEL 1963). Just as usual in any other angiospermous plant investigated to date, the cells of the outer tapetum contain one diploid nucleus during the premeiotic period (Fig. 2b). However, there is a different evolution of the cells of the inner tapetum. Exceedingly early one acytokinetic mitosis is brought about (Fig. 2a, note stage of anther wall development), which in some cells is completed and arrested during anaphase in others. Mitotic blocks prior to anaphase may occur but have not been observed. By this, most of the cells reach the tetraploid niveau. The cells enlarge and vacuolate. The premeiotic acytokinetic activity in a part of the tapetum is remarkable because there is considerable evidence that normally the start of the meiotic prophase is a prerequisite for polyploidization in the tapetum (CARNIEL 1963).

Sufficient information about the meiotic period is already available by the investigation of TSCHERMAK-WOESS (1967) in R. alectorolophus and R. minor, which is corroborated by our observations. Shortly after the meiotic prophase has started, in a part of the cells of the outer tapetum an acytokinetic but otherwise completely normal mitosis is accomplished, giving rise to an intermingled uni- and binucleate condition (Fig. 2c). Further mitotic activity completely fails but is replaced by one or two endomitoses. In the meiotic period, endomitosis also is the only way of polyploidization in the inner tapetum. During the meiotic period, under light microscope optics there are little reliable indications for the observed mitotic abnormalities of the inner tapetum prior to the meiotic period. This is due to the peculiar nuclear structure - large but few chromocenters and nearly invisible fine euchromatin - as well as to the equivocal appearance of nuclei which are situated tightly pressed against each other, and may be dumb-bell shaped or actually separated. However, our ultrastructu-

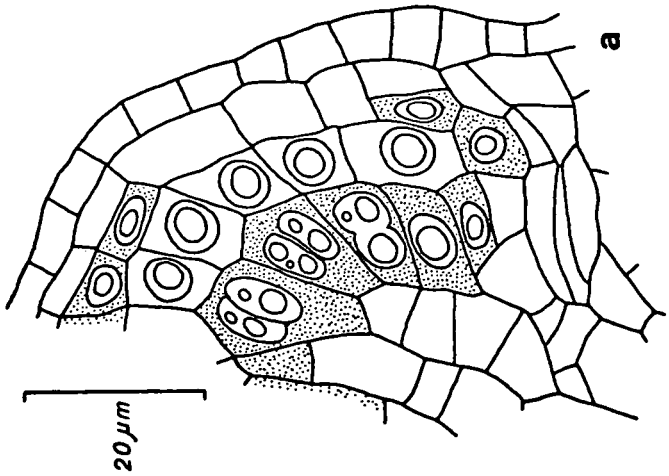
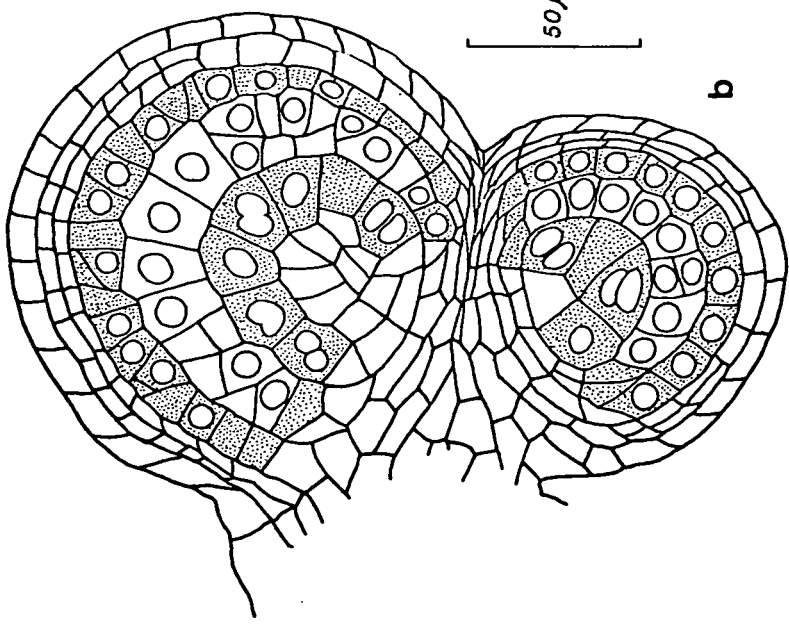


Fig. 2: Text see page 266.



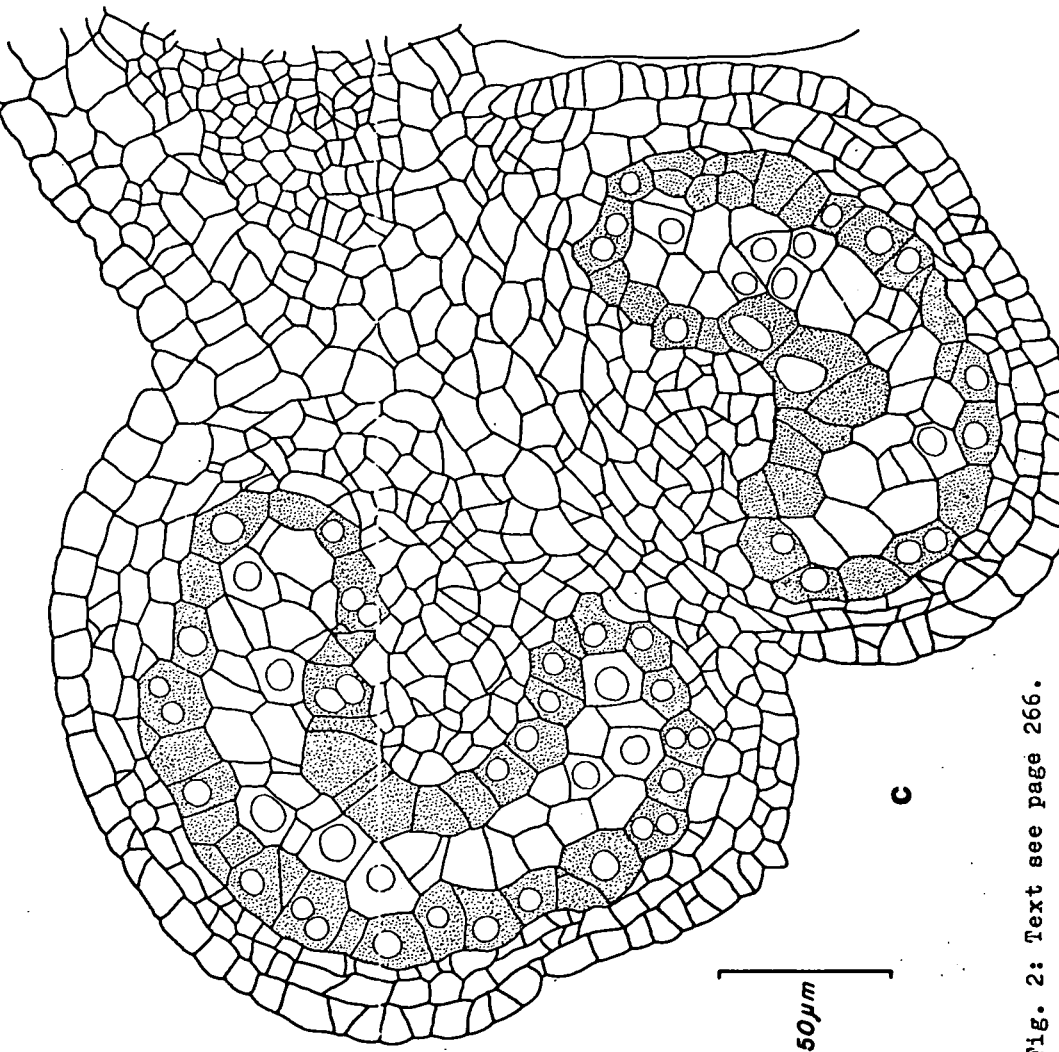


Fig. 2: Text see page 266.

Fig. 2. *Rhinanthus alectorolophus*, semi-thin sections of microsporangia (tapetal cells dotted). (a) early premeiotic period, outer tapetum only in part constituted, inner tapetum already binucleate resp. polyploid, (b) later stage of the premeiotic period, wall layers in part completed, outer tapetum uninucleate diploid, inner tapetum binucleate resp. polyploid, (c) meiotic period (zygotene), note intermingled uni- and binucleate condition of the outer tapetum.

Fig. 3. *Rhinanthus alectorolophus*, inner tapetum, meiotic period. (a)-(b) two prints from a serially sectioned restitution nucleus, note the single inner nuclear membrane (arrows) following the shortest diameter of the nucleus. L = cytoplasmic lacunas enclosed by the inner nuclear membrane only, NE = nuclear envelope, Mi = mitochondrion. - (a) 31000 x, (b) 35000 x.

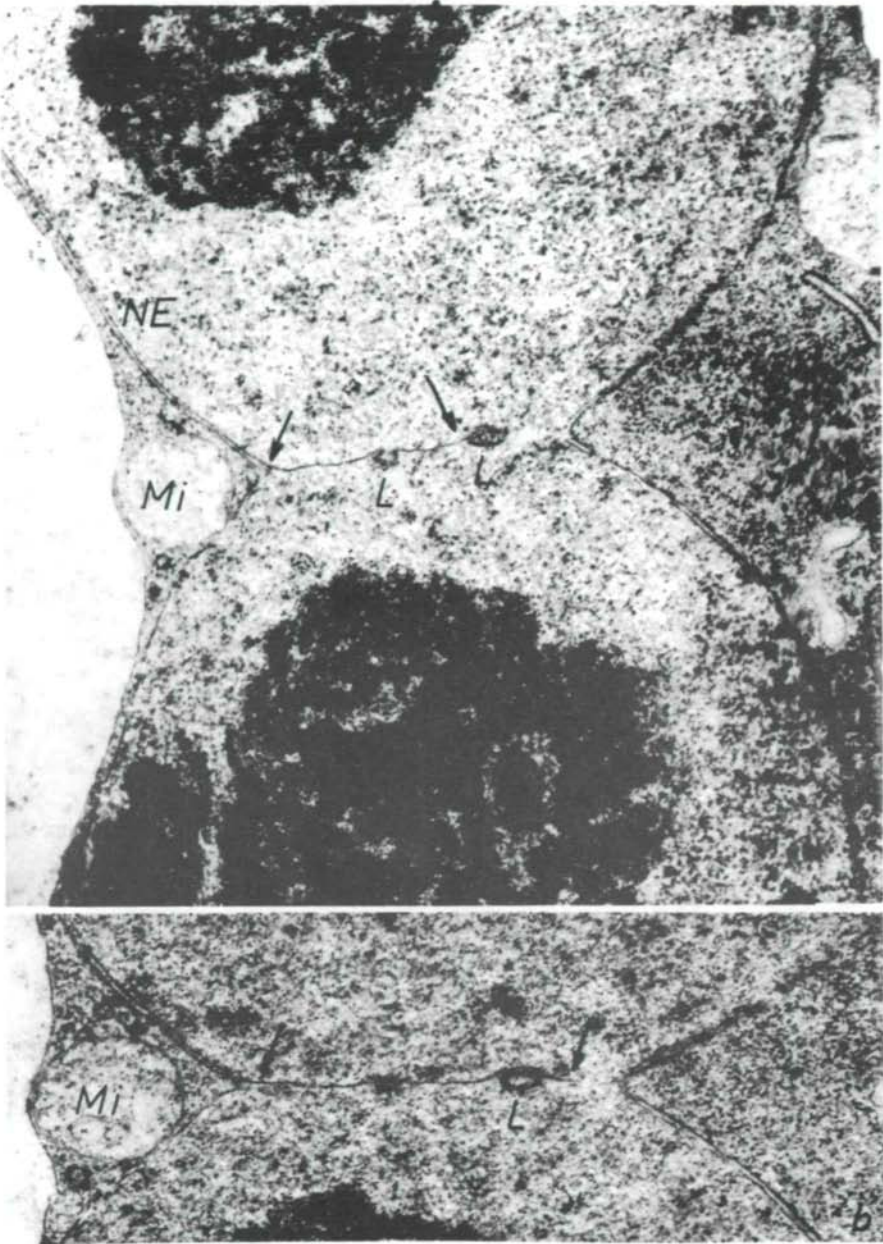


Fig. 3

ral investigation yielded clear evidence about the restitutorial nature of many nuclei of the inner tapetum, and only of those. Criteria were pronounced lobed nuclear shapes and dumb-bell nuclei, i.e. daughter nuclei which are only incompletely separated by the newly developed nuclear envelope. Nuclei as shown in Fig. 3 probably are brought about merely by failure of phragmoplast formation or by a collapse of this structure which is responsible for the definite separation of daughter nuclei (BAJER and MOLE-BAJER 1971). The opposite assumption, viz. real fusion of completed interphase nuclei, is as unnecessary as unlikely and would have no documented counterpart in tapetum cytology.

#### General aspects of tapetum differentiation.

The development of the tapetum in Rhinanthus is deviant and therefore noticeable for several reasons.

1. The dual origin of the tapetum is accompanied by an exceptional mitotic behaviour of the inner tapetum which contradicts the rule that polyploidization does not occur prior to the onset of meiosis.
2. During the meiotic period the outer tapetum first performs completely normal mitoses and then endomitoses, but no "intermediate" arrested mitoses.
3. The inner tapetum during the meiotic period follows the "new control" and grows by endomitosis despite of its different premeiotic development.

These peculiar features are tempting to speculate about the causes of the premeiotic different development and the tendency to identical development during meiosis. In our opinion, the premeiotic differences are causally connected with the crooked shape of the sporogenous cell mass.

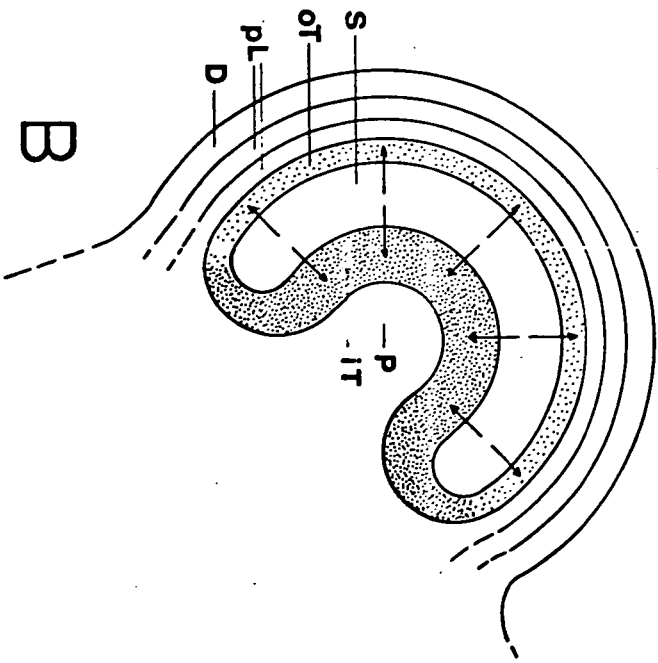
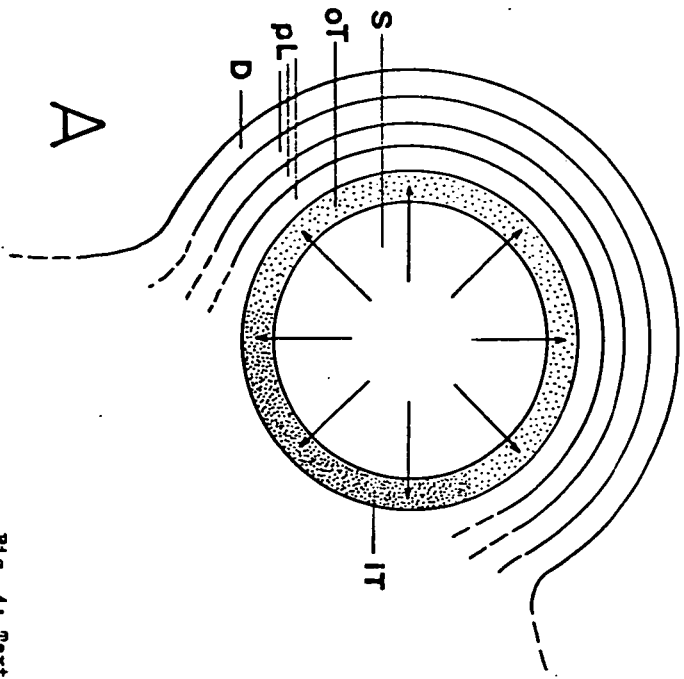


Fig. 4: Text see page 270.

Fig. 4. Hypothetical explanation of premeiotic tapetum development in plants with differently shaped archesporial complexes. Sketch of microsporangia in transverse section (tapetal layers spotted, the "inner tapetum" more tightly than the "outer tapetum").

A) The archesporium is disk-shaped in transverse section. Diffusing substantial factors which influence tapetum differentiation and development are equally concentrated around the sporogenous cell complex (note arrows). Therefore all tapetal cells develop in the same way. The vicinity of the vascular tissue (not shown) to the "inner tapetum" does not influence tapetum morphology.

B) The archesporium is reniform in transverse section. Diffusing factors are more concentrated in the inner tapetum than in the outer (note arrows). These factors are considered to inhibit spindle activity in the cell layer adjacent to the inner side of the sporogenous cell complex because of their high concentration. Mitotic activity continues, however, and multinucleate cells resp. restitution nuclei originate. The "placentoid" is a remnant of connective cells which are not used up for the tapetum. If all cells which are embraced by the sporogenous cells are transformed into tapetum, no placentoid is formed (for systematic significance of the "placentoid" see HARTL 1963).

D = epidermis, pL = parietal layers, oT = outer tapetum, S = sporogenous cell complex, iT = inner tapetum, P = placentoid.

Presumptively, the secondary archesporium and later the pollen mother cells provide substantial factors which are involved in or even regulate the cytological development of the tapetum. Then, the concentration of these factors in the neighbouring tissue layer largely depends on the shape of the sporogenous cell complex. If it is disk-shaped in the cross section (Fig. 4a), the diffusing factors must have the same concentration all around the archesporium, and cell development must be influenced equally. On the other hand, if the archesporial complex is of reniform cross section (Fig. 4b), as in *Rhinanthus*, the concentration at the inner side must be considerably higher than at the outer side. This diverse concentration is assumed to be the cause for the developmental differences of the outer and the inner tapetum. Concerning the question, whether the factor inhibits certain stages of cyto- and karyokinesis, or stimulates additional mitotic cycles, we assume an inhibitive effect because the first observable cytokinetic abnormalities occur in late parts of the mitotic cycle, and the connective tissue adjacent to the inner tapetum is just growing mitotically.

As stated above, generally there is a strict correlation between polyploidization and meiosis. This holds also true for *Rhinanthus*, in any case for the outer tapetum and also for the inner one regarding the mode of polyploidization during the meiotic period. According to our hypothesis a second and different factor is responsible for these cytological events. In our case this factor would determine "endomitosis" instead of mitosis, if present in a sufficient concentration. At an early stage of factor-release (early meiotic prophase) a certain part of the cells of the outer tapetum would still be able to perform mitosis unblocked until the concentration reaches a certain level. However, according to the crooked shape of the sporogenous cell mass, the inner tapetum already at the very beginning

would get a concentration of the factor sufficient to repress mitosis in favour of endomitosis.

This working hypothesis about the causes of diverse tapetum development may simplify, but has the advantage that evidence in favour or against it can be obtained simply by careful comparative ontogenetic and cytological analysis.

### Systematic significance of tapetum development in Pedicularieae.

As already indicated, considerable information on tapetum development in European Pedicularieae is now available. Although the ontogeny of sporogenous cells, wall layers, and tapetum is of the same kind throughout the tribe, the genera reveal different cytological patterns of tapetum development (listed and discussed in GREILHUBER 1974). However, 8 out of 10 genera are closely similar regarding their "type of tapetum", viz., they possess a "plurinu-cleate endopolyploid tapetum". This group comprises Bartsia, Odontites, Euphrasia, Bellardia, Rhinanthus, and the advanced parasites Tozzia, Lathraea, and (probably) Clandestina, - all members of the Euphrasia-group in Pedicularieae-Pediculariinae. Only Pedicularis and Melampyrum possess the more primitive type of tapetum which becomes polyploid by forming restitution nuclei. In this regard it is interesting to note that the isolated and primitive systematic position of Pedicularis is well established (HARTL 1969). On the other hand, Melampyrum commonly is placed within the Euphrasia-group of Pedicularieae-Pediculariinae next to Rhinanthus, which does not harmonize very well with its different type of tapetum. Also the tetrasporic embryo sac, the diploid endosperm (GREILHUBER 1973) and the kind of seed development (see HARTL 1974) may suggest to place Melampyrum more separately than before. Perhaps, its type of tapetum is an indication of a long time of separate evolution.



### Kurzfassung

#### Ultrastrukturelle und entwicklungsgeschichtliche Aspekte des "inneren Antherentapetums" von *Rhinanthus* (Scrophulariaceae)

Die vorliegende licht- und elektronenmikroskopische Studie befaßt sich mit der Entwicklung des Antherentapetums von *Rhinanthus*, wobei die Kernvorgänge und ihre Steuerung im Vordergrund stehen. Dabei wird dem Unterschied von Restitutionskernbildung und Endomitose besondere Bedeutung beigemessen. Während nämlich Restitutionskernbildung einen durch mannigfache Störungen und Hemmungen während der Mitose bedingten Polyploidisierungsvorgang bezeichnet, stellt die Endomitose im exakten Sinn einen streng geregelten abgekürzten Zellzyklus dar, bei dem keine prophasische Spiralisierung der Chromosomen erfolgt. Nach den bisherigen Befunden wird bei den allermeisten Angiospermen das Antherentapetum nach Einsetzen der meiotischen Prophase durch Restitutionskernbildung polyploid.

*Rhinanthus* weicht nun in mehrfacher Hinsicht von diesem weitverbreiteten Schema der Tapetum-Entwicklung ab:

1. Im Gegensatz zum äußeren Tapetum, das von seiner Differenzierung an bis zum Beginn der Meiose auf diploideinkerniger Stufe steht, wird das innere Tapetum extrem bald, nämlich schon zu einer Zeit, in der die Antherenwand noch perikline Teilungen durchführt, durch Restitutionskernbildung polyploid.
2. Während der meiotischen Periode erfolgt die Polyploidisierung nach dem seltenen Typ des "endopolyploiden Tapetums". Im inneren Tapetum kommt es zu einer Umstimmung von Restitutionskernbildung zur Endomitose. Im äußeren Tapetum läuft

anfangs in einem Teil der Zellen noch eine ungestörte Mitose ab, dann folgen nur mehr Endomitosen. Das Tapetum ist letztlich also "gemischt ein- und zweikernig endopolyploid".

Es wird - als Arbeitshypothese - eine Kausalerklärung der unterschiedlichen Entwicklung der beiden Teile des Tapetums in den beiden Phasen der Entwicklung geliefert. Es wird angenommen, daß die Kernvorgänge durch Stoffe, die vom Archespor abgegeben werden, gesteuert werden. Bedingt durch den stark gekrümmten nierenförmigen Querschnitt des Archespors müssen diese Stoffe (wahrscheinlich Zellteilungshemmer) an seiner Innenseite stärker konzentriert vorliegen als an seiner Außenseite, wirken innen daher auch früher als außen. Für die Umsteuerung von Mitose auf Endomitose während der frühen meiotischen Periode dürfte eine eigene stoffliche Determinante verantwortlich sein, die erst kurz nach Meiosebeginn gebildet wird.

Für die Richtigkeit der obigen Hypothese spricht, daß bei Pflanzen, deren Archespor annähernd kreisförmigen Querschnitt besitzt, inneres und äußeres Tapetum auf gleiche Weise und ohne zeitlichen Unterschied polyploid werden.

Ferner wird eine mögliche systematische Bedeutung der Tapetumentwicklung bei *Rhinanthus* diskutiert. Unter Auffassung von "Endomitose versus Restitutionskernbildung" während der meiotischen Periode als wesentliches Unterscheidungsmerkmal lassen sich Rhinanthus, Bartsia, Odontites, Euphrasia, Bellaria, Tozzia, Lathraea und Clandestina einerseits und Pedicularis und Melampyrum andererseits zu Gruppen zusammenfassen, wobei aber die beiden letzteren Gattungen sicherlich keine engere Verwandtschaftsgruppe bilden. Es wird jedoch ihre relative systematische Eigenständigkeit angenommen.

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